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RESEARCH ARTICLE

Impaired associative learning after chronic exposure to pesticides in young adult honey bees

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ABSTRACT

Neonicotinoids are the most widespread insecticides in agriculture, preferred for their low toxicity to mammals and their systemic nature. Nevertheless, there have been increasing concerns regarding their impact on non-target organisms. Glyphosate is also widely used in crops and, therefore, traces of this pesticide are likely to be found together with neonicotinoids. Although glyphosate is considered a herbicide, adverse effects have been found on animal species, including honey bees. *Apis mellifera* is one of the most important pollinators in agroecosystems and is exposed to both these pesticides. Traces can be found in nectar and pollen of flowers that honey bees visit, but also in honey stores inside the hive. Young workers, which perform in-hive tasks that are crucial for colony maintenance, are potentially exposed to both these contaminated resources. These workers present high plasticity and are susceptible to stimuli that can modulate their behaviour and impact on colony state. Therefore, by performing standardised assays to study sublethal effects of these pesticides, these bees can be used as bioindicators. We studied the effect of chronic joint exposure to field-realistic concentrations of the neonicotinoid imidacloprid and glyphosate on gustatory perception and olfactory learning. Both pesticides reduced sucrose responsiveness and had a negative effect on olfactory learning. Glyphosate also reduced food uptake during rearing. The results indicate differential susceptibility according to honey bee age. The two agrochemicals had adverse effects on different aspects of honey bee appetitive behaviour, which could have repercussions for food distribution, propagation of olfactory information and task coordination within the nest.

KEY WORDS: Olfactory learning, Responsiveness, Food uptake, Glyphosate, Imidacloprid, *Apis mellifera*

INTRODUCTION

Pests are a key contender in agricultural systems. Pest control using synthetic chemicals has increased substantially and is now a common and widely distributed practice in crop production (Hough, 2014; Ragsdale, 1999). Neonicotinoids constitute a chemical family that includes some of the most popular and extensively used insecticides. They act as neurotoxins by disrupting the nervous system, agonistically activating nicotinic acetylcholine receptors (Matsuda et al., 2001; Yamamoto, 1999). They can be applied as a seed dressing, through foliar spray or in the soil, and the plant then absorbs the chemical on germination or through its roots and distributes it systemically. This way, the insecticide reaches all plant tissues and can be traced in pollen, nectar and other plant fluids (Bonmatin et al., 2015; Mitchell et al., 2017). Another technology commonly used in agriculture is genetic modification of organisms, particularly the generation of insect-resistant and herbicide-tolerant crops (Raney, 2004). Glyphosate, a broad-spectrum herbicide used for its high effectiveness and low production cost, is a central character in this scenario. It inhibits an enzyme necessary for aromatic protein synthesis and, therefore, induces cellular disruption and eventual death. The pathway it acts on is present in plants, microorganisms and fungi, but not in animals (Amrhein et al., 1980). It is mainly applied by aerial or terrestrial spraying during tillage, or in a generalised manner in the case of herbicide-tolerant crops (Powles, 2008). This results in potential drift onto the air, soil and neighbouring crops (Matthews, 2008). Hence, traces of glyphosate are likely to be found together with neonicotinoids. Therefore, the combination of genetically modified crops and agrochemical use means that agroecosystems bare a mixture of chemicals, to which wild and domestic pollinators are exposed (Hladik et al., 2016; Traynor et al., 2016).

Even though neonicotinoids and glyphosate are vastly popular in agriculture, we must bear in mind that non-target organisms can still be harmed. This concern is extremely important when we take into account that 35% of world agricultural production depends on pollinators (Klein et al., 2007). The European Food Safety Authority has partially banned the use of certain neonicotinoids (European Commission, 2013). This measure was based on risk assessments for beneficial pollinators, which focused on three routes of exposure: residues in nectar and pollen of treated plants (Bonmatin et al., 2015; Mitchell et al., 2017), aerial particles emitted by pneumatic drilling machines (Girolami et al., 2012) and residues in guttation drops of treated plants (Tapparo et al., 2011). Adverse effects of glyphosate have been found on several animal species, including honey bees. Although the mode of action of this herbicide in insects is still unknown, parameters affected include development, reproduction, navigation, gustatory perception and olfactory learning (Balbuena et al., 2015; Helmer et al., 2015; Herbert et al., 2014; Thompson et al., 2014; Zanuncio et al., 2018). Therefore, it is reasonable to worry about its influence on pollinators. Glyphosate treatment has extended beyond agriculture to its use in gardening, forest engineering, illegal crop control and public transportation roads (Giesy et al., 2000). It has residual action, as dead plant tissues present herbicide traces and it can also be found in nectar and pollen of treated plants (Thompson et al., 2014).

An important crop pollinator is the European honey bee *Apis mellifera* L. Foraging honey bees can come into contact with these agrochemicals during their trips by flying through contaminated dust clouds, visiting treated plants or ones adjacent to treated crops,

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Received 22 December 2017; Accepted 27 February 2018

List of abbreviations

AM	accumulated mortality
CS−	unrewarded conditioned stimulus
CS+	rewarded conditioned stimulus
DI	discrimination index
PER	proboscis extension response
TIU	total individual uptake

or gathering water, nectar or pollen that contains pesticide traces. Furthermore, as *A. mellifera* is a eusocial insect, colony survival depends on collective tasks. Thus, it is important to take into account exposure of bees that remain inside the hive. Once a forager returns to the hive, she can contaminate her mates through body contact, when sharing food or through the collected resources that are eventually stored in cells (DeGrandi-Hoffman and Martin, 1993; Grüter and Farina, 2007). The high connectivity among colony mates within a beehive allows information transfer regarding resources. This is crucial for individuals that remain inside the hive, such as young workers, as they receive cues from outside, especially those perceived through taste and smell (Farina et al., 2007). Young workers perform in-hive tasks that guarantee colony care and maintenance (Seeley, 1982). They also present changing physiology and anatomy (Amdam et al., 2004; Masson and Arnold, 1987) and exhibit high physiological and behavioural plasticity (Arenas et al., 2013). Recent reports confirm the presence of neonicotinoids and glyphosate traces in honey samples (Mitchell et al., 2017; Rubio et al., 2014), indicating that the young adults and brood come into contact with these chemicals within the colonies. For these reasons, we consider young workers to be critical for assessing the effect of agrochemicals on the honey bee *A. mellifera*.

Honey bees constitute successful models for behaviour studies under full control of the experimenter. Different protocols are used to analyse honey bees' sensory skills, cognitive abilities, malaise behaviour, etc. For young worker bees, perception and cognitive skills are essential as they require the integration of these processes to carry out their activities. This is why we studied sensory and cognitive abilities in young adults. In the case of associative learning, different conditioning procedures reveal cognitive processes of a different nature (Giurfa, 2003). For this reason, to evaluate the actual impact of an agrochemical on behaviour, we thought it worth addressing this question through different bioassays. With this in mind, we considered two learning protocols – absolute and differential conditioning – as well as a gustatory responsiveness assay to assess the effect of realistic concentrations of a neonicotinoid insecticide, imidacloprid, and the herbicide glyphosate on these variables. As honey bees are exposed to multiple pesticides in an agricultural scenario, we considered a dual exposure to these pesticides. Also, as young honey bees present different physiological and anatomical stages, we focused on several ages with the aim of picking up differential effects according to age.

MATERIALS AND METHODS

Animal rearing and experimental series

The study was carried out during the summer–autumn seasons (December to April) of 2014 to 2016. European honey bees (*A. mellifera*) were obtained from the apiary at the experimental field of the Universidad de Buenos Aires, Argentina. Sealed brood frames were selected and kept in an incubator in the laboratory at 32°C and 55% relative humidity. Every 24 h, newly emerged workers were collected in groups of 60–80 individuals and confined in wooden boxes (10 cm×10 cm×10 cm) with a metallic mesh on one side and a plastic door on the opposite side; these were kept in another incubator at 30°C, and were supplied with syrup (50% w/w sucrose solution) and pollen *ad libitum*. Syrup was replaced every 2 days and dead bees were removed upon appearance. Every day, we counted the number of dead bees and measured the amount of syrup consumed in each cage. Daily syrup consumption was calculated relative to the number of live bees to obtain a measure of the total individual uptake (TIU). Accumulated mortality (AM) was calculated as the total number of dead bees throughout the rearing period. TIU and AM were calculated for each treatment and for each rearing period.

Chronic exposure was achieved by adding pesticides to food throughout the entire rearing period. Treatments were control (syrup only), imidacloprid, glyphosate and imidacloprid+glyphosate. Imidacloprid (99.9% purity, Sigma-Aldrich, St Louis, MO, USA) and glyphosate (99.7% purity, acid form, Sigma-Aldrich) stock solutions were prepared with distilled water and had a final concentration of 4 mg l^{-1} and 100 mg l^{-1}, respectively. These were dissolved in syrup each time the treatment was replaced. Final concentrations were 1 μg l^{-1} for sublethal and field-realistic imidacloprid values (Cresswell, 2011) and 2.5 mg l^{-1} for sublethal and worst-case scenario glyphosate values (Giesy et al., 2000; Herbert et al., 2014). The final concentration of each agrochemical was the same in the mixture as in the corresponding pure treatment. Cages were randomly assigned to each treatment, maintaining a balanced sample size per treatment.

Young workers were tested in laboratory bioassays when they were 5, 9 or 14 days old. At that time, each bee was captured from its cage, anaesthetised at −4°C and harnessed in a carved pipette tip, which restrained body movement but allowed it to freely move its mouthparts and antennae. Afterwards, bees were kept in the incubator for an hour and a half to minimise stress caused by handling (Matsumoto et al., 2012; Mengoni Goñalons et al., 2016).

Sucrose responsiveness

Honey bees extend their proboscis as a reflex in response to an appetitive stimulus such as nectar (Frings, 1944). To study the sucrose response threshold, bees were stimulated with sucrose solutions of increasing concentration (0.1, 0.3, 1, 3, 10, 30 and 50% w/w) by touching their antennae (Page et al., 1998). Bees were lined up in groups of 30–40 individuals and tested sequentially for each concentration. Before presentation of each sucrose solution, all bees were tested for their response to water (0%). This controlled for potential effects of repeated sucrose stimulation that could lead to increased sensitization or habituation, as well as assuring that extension of the proboscis was not due to thirst. The interstimulus interval between presentation of water and the sucrose solution was 4 min. At the end of the experiment, a gustatory response score was obtained for each bee, based on the number of sucrose concentrations to which the bees responded (values from 1 to 7). If a bee failed to respond to one sucrose concentration in the middle of a response series, this 'failed' response was considered to be an error and the bee was deemed to have responded to that concentration as well. A bee that did not respond to any of the sucrose concentrations was excluded from further analyses. In addition, those bees that responded to all sucrose concentrations and all presentations of water were excluded from analyses as they appeared not to be able to discriminate between sucrose solution and water.

Olfactory conditioning

The proboscis extension response (PER) to sucrose solution is an unconditioned response and can be conditioned in a classical way

(Bitterman et al., 1983; Takeda, 1961). In olfactory conditioning, bees are trained to associate an initially neutral odour (conditioned stimulus, CS) with a sucrose reward and finally exhibit a PER towards the odour alone (conditioned response). In this study, two types of conditioning procedure were performed, absolute and differential (Bitterman et al., 1983; Takeda, 1961). In the case of the former, a single odour was paired with sucrose solution. However, this procedure does not explicitly distinguish odour–reward association from other non-associative processes. In the case of the latter, one odour was rewarded (rewarded conditioned stimulus, CS+) and another was presented alone (non-rewarded conditioned stimulus, CS−). This procedure constrains the bee to discriminate between the two conditioned stimuli in terms of their association with the reward and, therefore, provides a within-group control for the associative nature of the bee's performance (Bitterman et al., 1983).

Only bees that extended their proboscis to syrup (unconditioned response towards the unconditioned stimulus) were used for olfactory conditioning. During conditioning, the harnessed bee was placed between a device that produced a constant airflow and an extractor fan which removed released odours. The airstream ($2.5\ \mathrm{ml\ s^{-1}}$) was delivered to the head of the bee from a distance of 2 cm. Bees that responded to the mechanical air stimulus were discarded. The rewarded odour was 1-hexanol and the unrewarded odour was nonanal. Odours were delivered when the airflow was redirected, by means of an electric valve, to pass through a syringe containing a 30 mm×3 mm piece of filter paper impregnated with 4 µl of the pure odour. Odour was delivered for a period of 6 s and the reward was presented during the last 3 s by touching the antennae with syrup and then allowing the bees to feed. A conditioned response was computed if the bee fully extended its proboscis during the first 3 s of odour delivery. One trial lasted for 39 s and was composed of 16 s of clean airflow, 6 s of odour and 17 s of clean airflow. Simple training consisted of 5 trials whereas differential training consisted of 5 rewarded trials and 5 non-rewarded ones presented in a pseudo-randomised fashion. Inter-trial interval was 15 min. Bees that presented spontaneous PER towards the odour were discarded from analysis. For performance analysis of the differential conditioning, a discrimination index (DI) was defined. A bee that extended its proboscis towards the CS+ but not the CS− was considered to have discriminated between the CS (DI=1). A DI was calculated for each trial pair of the training phase and for the single test trial.

A period of 20 min was introduced between the last trial and the testing phase, which consisted of non-rewarded presentation of the conditioned stimuli used in the training phase of each protocol. After the testing phase, the unconditioned response was verified and bees that did not present it were discarded from further analyses.

Statistical analysis

The effects of factors on all response variables were assessed by means of generalised linear models or generalised linear mixed models. The latter case included random factors. Models were fitted in R (R Foundation for Statistical Computing, Vienna, Austria) using the glm function for generalised linear models and the glmer function of the lme4 package (Bates et al., 2015) for generalised linear mixed models. The saturated models included all factors and covariates of interest (Table S2). Alternative models – which differed in complexity – were assessed and compared, and one was chosen depending on its Akaike information criterion value. *Post hoc* comparisons using contrast matrixes were performed with the glht function of the R package multcomp (https://CRAN.R-project.org/package=multcomp).

RESULTS

Mortality

Honey bees were reared in wooden boxes in the laboratory from adult emergence until days 5, 9 and 14. Imidacloprid, glyphosate or both were added to the sugared syrup throughout the rearing period. Dead bees and food uptake were measured daily. After each rearing period, we calculated the AM as the total number of dead bees. Addition of agrochemicals did not affect AM after any of the exposure periods (Fig. S1; AM_5~cage, AM_9~cage, AM_{14}~cage, where the subscript indicates the number of days). Most of AM values were less than 20%.

Food uptake

Daily syrup consumption was calculated relative to the number of live bees to obtain a measure of the TIU. Imidacloprid presence had no influence on syrup uptake. In contrast, glyphosate presence affected TIU after all exposure periods, depending on the season the experiment took place in (TIU_5~glyphosate×season, TIU_9~glyphosate×season, TIU_{14}~glyphosate×season). Bees reared in 2014 that were offered food combined with glyphosate, after 5, 9 or 14 days, consumed less syrup than bees reared in control or imidacloprid cages (Fig. 1). In assays performed in the years 2015 and 2016, glyphosate presence did not affect TIU (Fig. S2). For behavioural assay analyses, TIU was included as a covariate in the saturated model.

Gustatory responsiveness

At 5, 9 or 14 days of age, honey bees were tested for their sucrose responsiveness. They were presented with sucrose solutions of increasing concentration and assigned a score defined as the sum of PERs throughout the procedure (Page et al., 1998). Syrup consumption had no effect on sucrose responsiveness. Agrochemical treatment had an influence on responsiveness, but its effect did not depend on honey bee age (score~imidacloprid×glyphosate+age+cage). Honey bees that fed on sucrose solution plus either agrochemical had lower scores than control bees, implying a rise in sucrose response thresholds. The presence of an additional agrochemical did not modify sucrose responsiveness in comparison to the presence of either agrochemical alone (Fig. 2, left). In contrast, 14 day old bees had lower scores than younger bees, although statistical analysis only confirmed this difference in comparison to 9 day old bees; in other words, their sucrose sensitivity was lower (Fig. 2, right).

Absolute conditioning

At 5, 9 or 14 days of age, honey bees were trained to associate a pure odour with a sucrose reward via absolute olfactory conditioning. Syrup uptake during rearing did not influence the conditioned response during the training phase. Treatment effect depended on honey bee age (PER~imidacloprid×glyphosate×age+trial+cage+bee). Bees aged 9 days and older were not affected by the agrochemicals (Fig. 3, middle and right). However, 5 day old bees that had been feeding on sucrose solution with imidacloprid alone showed a diminished performance during the training phase. Surprisingly, bees that had been reared with imidacloprid together with glyphosate showed a different performance from the first group and similar to that of the control group. In other words, the effect of imidacloprid was no longer observed in the presence of glyphosate (Fig. 3, left). Treatment had no effect on the conditioned response in the testing phase (data not shown; PER~TIU+cage).

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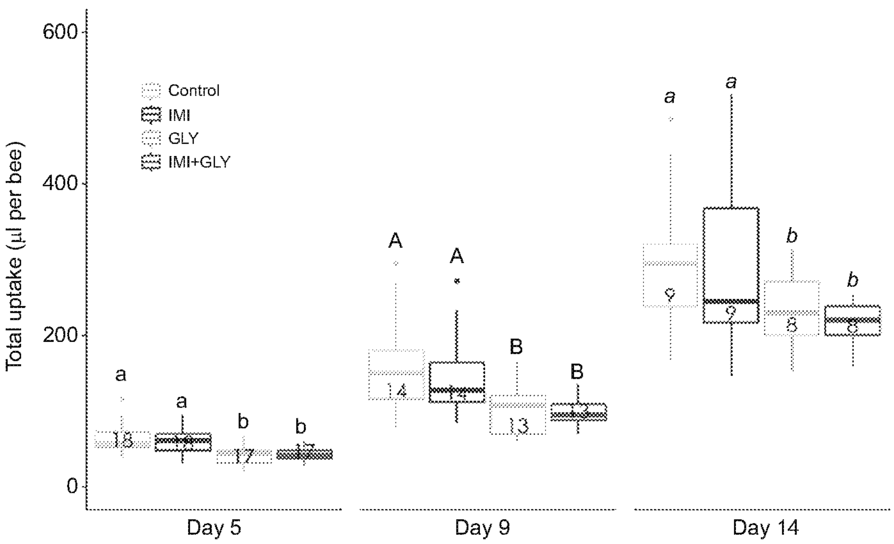

Fig. 1. Effect of chronic exposure to imidacloprid, glyphosate or both on syrup uptake by young honey bees. Total volume of sucrose solution ingested per bee after 5, 9 or 14 days of laboratory rearing in a cage that offered sucrose solution alone (control), with imidacloprid (IMI), with glyphosate (GLY) or with both agrochemicals. Only data from the year 2014 are shown. Thick line, box and whiskers represent median, inter-quartile range and data range excluding extreme data (points), respectively. Numbers inside boxes indicate sample size. Letters indicate significant differences. Minimal adequate models: TIU_5~glyphosate×season, TIU_9~glyphosate×season, TIU_{14}~glyphosate×season, where TIU is total individual uptake and subscript indicates number of days.

Differential conditioning

At 5, 9 or 14 days of age, honey bees were trained to associate a pure odour with a sucrose reward and distinguish it from another that was presented alone, in a differential olfactory conditioning procedure. A DI was calculated based on the responses towards both odours. Syrup uptake during rearing had no effect on learning performance during training. Treatment effect on this ability depended on honey bee age (DI~imidacloprid×glyphosate×age+trial pair+cage+bee). Five day old bees exposed to imidacloprid showed diminished odour discrimination compared with control bees. Glyphosate did not affect DI. Double agrochemical exposure yielded an intermediate discrimination level, as this group was statistically undefined between the imidacloprid group and the glyphosate group. In addition, it was not different from the control group (Fig. 4, left). Nine day old bees exposed to either pesticide showed a decreased performance (Fig. 4, middle). In the testing phase, no variable affected DI (data not shown; DI~cage). To summarise, imidacloprid affected differential olfactory learning acquisition in 5 and 9 day old bees and glyphosate only acted on 9 day old bees. Older bees were unaffected by the pesticides.

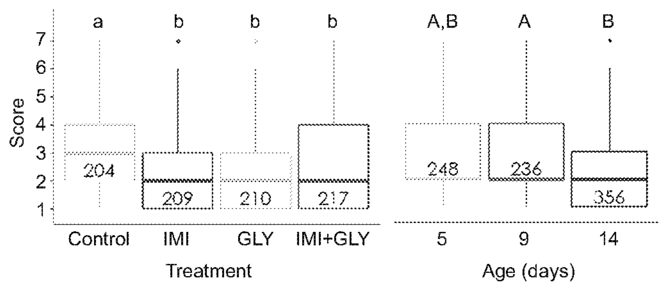

Fig. 2. Effect of chronic exposure to imidacloprid, glyphosate or both on young honey bee sucrose responsiveness. The gustatory response score of 5, 9 or 14 day old bees reared in cages that offered sucrose solution alone (control), with imidacloprid, with glyphosate or with both agrochemicals. Thick line, box and whiskers represent median, inter-quartile range and data range excluding extreme data (points), respectively. Numbers inside boxes indicate sample size. Letters indicate significant differences between the specified treatments (lowercase) or between the specified ages (uppercase). Minimal adequate model: score~imidacloprid×glyphosate+age+cage.

DISCUSSION

According to the evidence gathered in this study, we can conclude that the imidacloprid and glyphosate concentrations used had sublethal effects on young honey bees. Imidacloprid negatively affected gustatory responsiveness and olfactory learning, depending on honey bee age. Glyphosate reduced sensitivity to sucrose and, in one case, olfactory discrimination. Some results suggest an interaction between pesticides, but there was no clear evidence of this.

Imidacloprid and glyphosate concentrations are sublethal

The imidacloprid concentration chosen for this study is 10 times lower than the maximum field-realistic concentration found in nectar (Cresswell, 2011) and 1000 times lower than the average concentration found in a world collection of honey samples from apiaries and commercial enterprises (Mitchell et al., 2017), which gives an idea of the residues a young honey bee would encounter. Additionally, the concentration was sublethal as exposure of honey bee groups resulted in 10% average mortality (Cresswell, 2011) and, in our study, it did not induce differential mortality in comparison with the control group. One study found that exposure of foraging age honey bees to 2.56 $\mu g\ l^{-1}$ of imidacloprid for 3 days resulted in 20% mortality (Williamson and Wright, 2013). Another study used hive bees and found that a 10 day exposure to 1 $\mu g\ l^{-1}$ of imidacloprid yielded 40% mortality (Suchail et al., 2001); in that study, bees were reared in groups of 30, a small number for *in vitro* rearing, which probably contributed to increased mortality (Williams et al., 2015). The glyphosate concentration used – which is equivalent to 2.08 $mg\ kg^{-1}$ – is similar to the highest found in agricultural environments (Giesy et al., 2000) but we cannot ensure that is nest-realistic as there is a lack of studies addressing this question. One study exposed honey bee hives to a *Phacelia tanacetifolia* plantation sprayed with 7.2 $g\ kg^{-1}$ glyphosate in water. One day after treatment, glyphosate residue in nectar collected from foragers was 31.3 $mg\ kg^{-1}$. Six days after that, residue in nectar collected from hive combs was 0.99 $mg\ kg^{-1}$ (Thompson et al., 2014). This concentration is lower than the one used in our study, but it is the same order of magnitude. Another study measured residues in commercially produced honey and found that 70% had average glyphosate concentrations of 0.064 $mg\ kg^{-1}$ (Rubio et al., 2014). This is a much lower

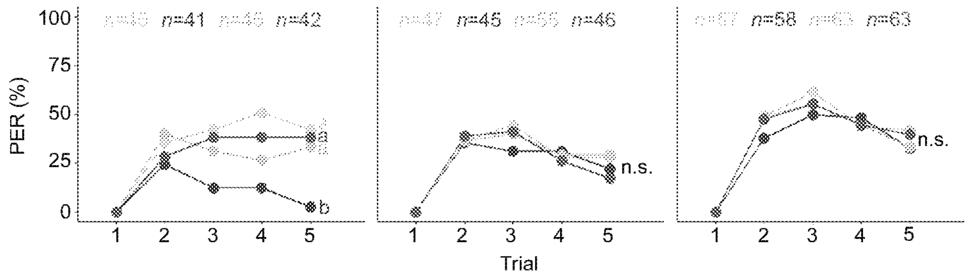

Fig. 3. Effect of chronic exposure to imidacloprid, glyphosate or both on absolute olfactory conditioning of young honey bees. The percentage of bees that extended their proboscis (proboscis extension response, PER) towards the conditioned stimulus (CS) in the training phase of an absolute classical conditioning protocol. Bees were (from left to right) 5, 9 or 14 days old and had been reared in cages that offered sucrose solution alone (control, green), with imidacloprid (purple), with glyphosate (orange) or with both agrochemicals (pink). *n*, sample size. Letters indicate significant differences between the specified treatments; n.s., non-significant difference between treatments. Minimal adequate model: PER~imidacloprid×glyphosate×age+trial+cage+bee.

concentration, but as samples were taken from commercial honey purchased in markets and not from honey combs, we do not know the actual concentration hive bees would have been exposed to.

The imidacloprid concentration was also sublethal as mortality after 14 days was 6% in average, and not higher than 20%. A previous study that used the same concentration of imidacloprid during the same rearing period observed 24% mortality (Herbert et al., 2014).

A combined effect of imidacloprid and glyphosate is unclear

The lack of independence or addition of the effects of two compounds denotes interaction between them. Typically, toxicological studies that address this property include several concentrations of each compound (Jonker et al., 2005). However, our aim was to assess the existence of a simple interaction between field-realistic concentrations of the pesticides. The deficit observed in sucrose sensitivity and differential olfactory learning assays due to dual exposure (Figs 2 and 3, respectively) was similar to that following exposure to imidacloprid or glyphosate alone. Therefore, in these cases, there is no evidence of an interaction between the insecticide and the herbicide, or even addition of the effects.

By contrast, 5 day old bees that consumed both imidacloprid and glyphosate together with their food did not show the same diminished response of bees that were exposed to imidacloprid alone during absolute conditioning (Fig. 3). This is consistent with an antagonistic interaction, where glyphosate presence masks the effect of imidacloprid. It is noteworthy that bees reared with glyphosate – alone or together with imidacloprid – consumed less food. Therefore, bees reared with imidacloprid plus glyphosate inevitably accumulated less imidacloprid than those exposed to imidacloprid alone. Although TIU was excluded from the minimal adequate model explaining PER towards the CS, we cannot discard a biological effect that passes undetected in a statistical analysis due to lack of power.

Honey bees feed less on syrup with glyphosate traces

Glyphosate decreased sucrose solution consumption during rearing in the year 2014 (Fig. 1). This result was unclear in the following seasons (Fig. S2). Low sample size could explain the lack of statistical significance in these cases. Nevertheless, we must bear in mind that environmental conditions and the bee's genetics can account for different tendencies between seasons. However, we cannot ignore that the reduced food uptake could be due to different phenomena, such as a direct rejection of glyphosate, a reduced sweet taste of the glyphosate–syrup mixture (Desmedt et al., 2016) or the induction by glyphosate of some kind of malaise which makes honey bees feed less. We cannot discriminate between these hypotheses because exposure was chronic and no alternative glyphosate-free food was offered. For the first, forager bees continued visiting a food source offering sucrose solution with 2.5 mg l^{-1} glyphosate (Herbert et al., 2014), which would indicate no rejection. Nevertheless, physiological state and motivation of

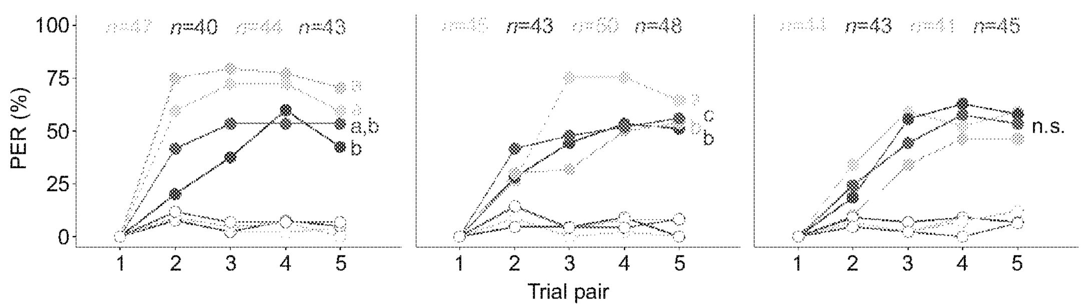

Fig. 4. Effect of chronic exposure to imidacloprid, glyphosate or both on differential olfactory learning of young honey bees. Percentage of bees that extended their proboscis towards the rewarded conditioned stimulus (CS+, filled circles) and the unrewarded conditioned stimulus (CS−, open circles) during the training phase of a differential classical conditioning protocol. Bees were (from left to right) 5, 9 or 14 days old and had been reared in cages that offered sucrose solution alone (control, green), with imidacloprid (purple), with glyphosate (orange) or with both agrochemicals (pink). *n*, sample size. Letters indicate significant differences between the specified treatments; n.s., non-significant differences between treatments. Statistical analysis was performed using a discrimination index (DI), based on the bee's ability to discriminate between the CS+ and the CS− in each trial pair. Minimal adequate model: DI~imidacloprid×glyphosate×age+trial pair+cage+bee.

foragers could constrain them to continue exploiting a non-palatable food source (Ayestaran et al., 2010). Regardless of the cause, the fact that bees exposed to glyphosate consumed less sucrose solution added a source of variation. This is why TIU was considered a predictor variable in behavioural analyses. Nevertheless, we found that differences were due to herbicide exposure and not to reduced food uptake.

Imidacloprid and glyphosate decrease sucrose responsiveness

Both imidacloprid and glyphosate had a negative effect on gustatory perception. Honey bees of all ages exposed to pesticides were more likely to extend their proboscis to higher sucrose concentrations than control bees, which indicates a higher response threshold. The effect of glyphosate on sucrose responsiveness was previously observed in a study that exposed newly emerged honey bees to 2.5 or 5 mg l^{-1} glyphosate for 15 days. That study showed that the glyphosate-exposed bees had lower scores than control bees (Herbert et al., 2014). The effect of chronic imidacloprid exposure on gustatory responsiveness had not been addressed until this study. However, if we consider the total amount of imidacloprid assimilated as a dose (Table S1), the effect of chronic imidacloprid is similar to the effect of acute imidacloprid (Eiri and Nieh, 2012).

Pesticide effect on gustatory perception was age independent, which indicates that their mechanism of action on this pathway does not depend on the honey bee's physiology or on exposure time. Regardless of which treatment they received, 14 day old bees exhibited lower sucrose sensitivity than 9 day old bees. This was unexpected as this attribute increases with age (Scheiner et al., 2004).

Imidacloprid and glyphosate impair olfactory learning abilities

Imidacloprid negatively affected performance in the absolute conditioning procedure only in the case of 5 day old bees. Bees of this age that incorporated imidacloprid in their diet showed an impoverished acquisition dynamic, where the probability of PER towards the CS started decreasing from the second trial and ended near 0 at the end of the training phase. This detrimental effect on acquisition was found in another study that exposed foragers to 2.56 μg l^{-1} imidacloprid for 4 days (Williamson and Wright, 2013). Interestingly, our 14 day old bees – an age close to foraging – were not affected by imidacloprid. This may be because our bees accumulated 0.25 ng of imidacloprid whereas bees in the other study incorporated a total 1.3 ng of imidacloprid together with their food. It is noteworthy that the influence of imidacloprid on performance in this procedure does not necessarily imply an effect on odour–reward association as non-associative processes could also be affected (Menzel, 1999). In this sense, differential conditioning serves as a within-group control (Bitterman et al., 1983). As the PER must be restricted to the rewarded odour, it discards an effect of exposure to the CS independently of its pairing and provides evidence of an associative process. As younger bees exposed to imidacloprid performed poorly in this protocol as well, we can conclude that imidacloprid affects associative processes in 5 day old bees.

Nine day old bees were susceptible to imidacloprid and glyphosate when examined in the differential conditioning procedure, but not in the absolute conditioning procedure. This protocol tests the ability to associate an odour with a reward as well as the capacity to distinguish it from another that is not linked to a reward. Therefore, an impoverished performance in differential conditioning could be due to bees confusing the rewarded conditioned stimulus (CS+) with the unrewarded one (CS−) or to a non-specific association between the CS and the reward (Matsumoto et al., 2012). Imidacloprid was found to reduce perceptual distance between odour representations in the glomeruli of the antennal lobes (Andrione et al., 2016). This generalisation phenomenon could partly explain why the effect of imidacloprid on 9 day old bees is evidenced only in the differential conditioning assay. In terms of the effect of glyphosate, its mechanism of action on olfactory perception is unknown. Nevertheless, its negative effect on olfactory associative learning was observed in the past in young adult laboratory bees (Herbert et al., 2014). Even forager honey bees exposed to acute glyphosate doses displayed weakened cognitive capacities needed to retrieve and integrate information for successful foraging (Balbuena et al., 2015; Herbert et al., 2014). It is worth mentioning that the differential conditioning experiment detected pesticide effects that the absolute conditioning experiment failed to reveal. Therefore, it emerges as a more sensitive and effective protocol when reviewing toxicity in terms of honey bee cognitive abilities.

Contrary to sucrose responsiveness, the agrochemical effect on learning was age dependent. As cage-reared bees only defecate if they are released in a flying arena (Núñez, 1970), the magnitude of the effect should increase with exposure time as a result of substance build up. However, we observed the opposite, where the effect size of imidacloprid decreased with age. Therefore, we can attribute differential effects to honey bee age and not time of exposure to the compound. This age dependency could be a reflection of differences in detoxification. In fact, honey bees metabolise imidacloprid through oxidation processes (Suchail et al., 2004). From this point of view, two events could explain our results. On the one hand, given that honey bees exhibit physiological and anatomical specialisations according to age (Winston, 1987), younger honey bees could have a weaker detoxification system than older ones. On the other hand, as younger bees consumed less imidacloprid (Table S1), this small dose could be insufficient to trigger a detoxification process (Nagata et al., 1998).

Adverse effects on young bees influence the whole colony

Young honey bees are colony members which exhibit high behavioural and physiological plasticity (Arenas et al., 2013) and which perform relevant tasks that guarantee nest maintenance and colony care (Lindauer, 1952; Seeley, 1982). Perception and learning skills are essential for task execution. Also, experiences from early adulthood can shape later behaviour and affect tasks performed in the future (Arenas et al., 2013). These include those performed inside the nest, such as food processing, and foraging for resources outside. A learning deficit due to difficulties in establishing odour–reward associations would affect propagation of olfactory information (Ramirez et al., 2010). Sucrose sensitivity of pre-foraging bees is associated with their foraging behaviour (Pankiw et al., 2004). Therefore, detrimental effects on gustatory perception and olfactory learning would impact overall nectar distribution, which would imply that the hive could face the end of the season with limited and potentially contaminated resources.

Acknowledgements
We thank Héctor Verna for technical assistance during the experimental period. We also thank the CONICET for support.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.M.G., W.M.F.; Methodology: C.M.G., W.M.F.; Validation: C.M.G.; Formal analysis: C.M.G.; Investigation: C.M.G., W.M.F.; Resources: C.M.G., W.M.F.; Data curation: C.M.G., W.M.F.; Writing - original draft: C.M.G., W.M.F.; Writing - review & editing: C.M.G., W.M.F.; Supervision: W.M.F.; Project administration: W.M.F.; Funding acquisition: W.M.F.

Funding
This study was partly supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), Universidad de Buenos Aires and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) to W.M.F.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.176644.supplemental

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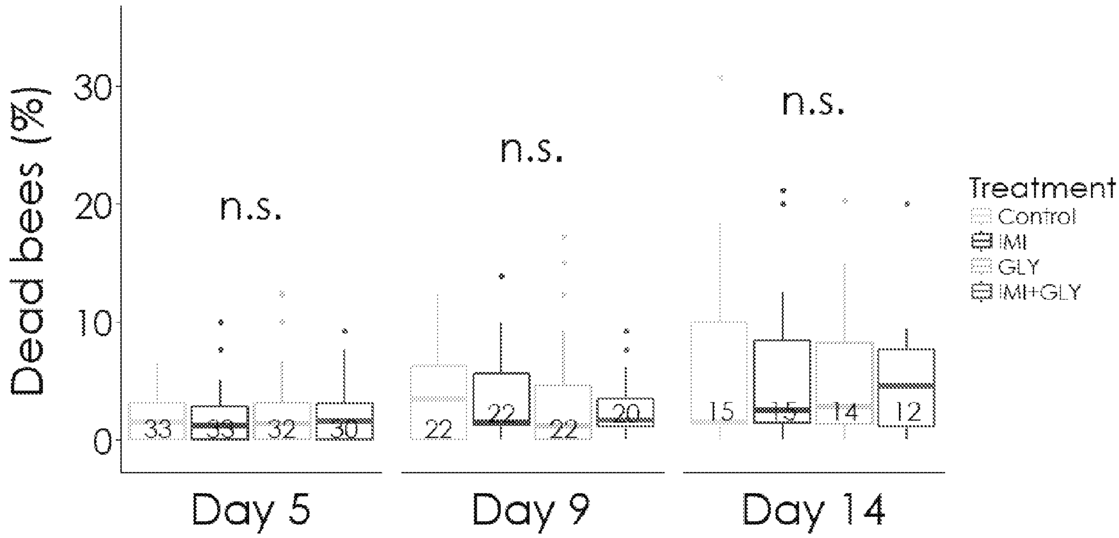

Figure S1. Effect of chronic exposure to IMI, GLY or both on honey bee mortality. Percentage of dead bees accumulated until days 5, 9 or 14 after laboratory rearing in cages that offered sucrose solution alone (Control, green), with IMI (purple), with GLY (orange) or with both agrochemicals (pink). Thick line, box and whiskers represent median, inter-quartile range and data range excluding extreme data (points), respectively. Numbers inside boxes indicate sample size. "n.s." stands for non-significant differences between treatments.

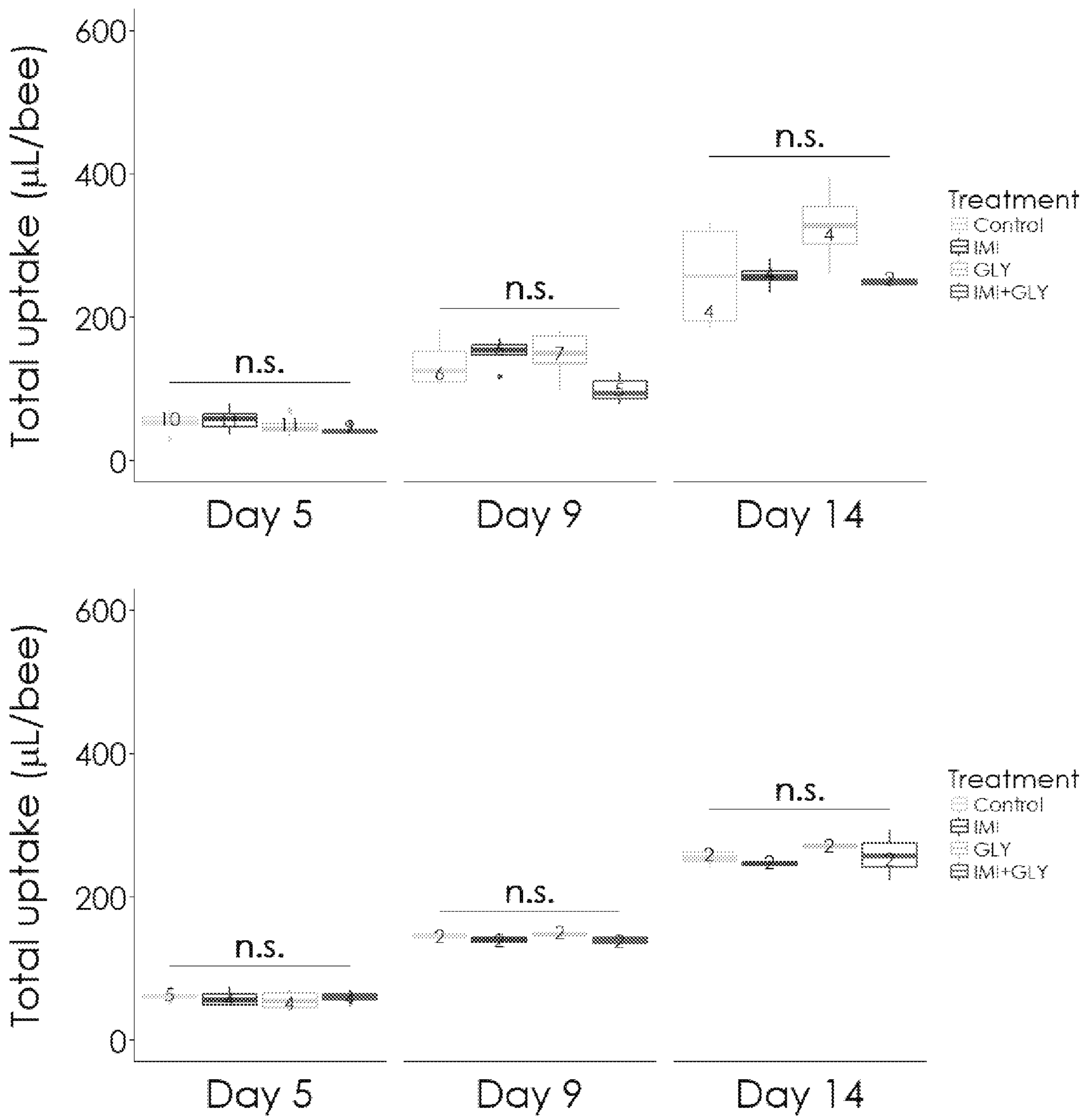

Figure S2. Effect of chronic exposure to IMI, GLY or both on syrup uptake. Total sucrose solution volume ingested by one bee after 5, 9 or 14 days of laboratory rearing in a cage that offered sucrose solution alone (Control, green), with IMI (purple), with GLY (orange) or with both agrochemicals (pink). Data belong to experiments performed in the years 2015 (top) and 2016 (bottom). Thick line, box and whiskers represent median, inter-quartile range and data range excluding extreme data (points), respectively. Numbers inside boxes indicate sample size. The asterisks indicate significant differences between the specified treatments.

Table S1. Total pesticide uptake during rearing. “n” indicates the number of cages.

Exposure time (days)	Treatment	n	Total IMI (ng/bee)	Total GLI (µg/bee)
5	IMI	27	0.057	-
	GLI	28	-	0.112
	IMI + GLI	26	0.044	0.110
9	IMI	20	0.137	-
	GLI	22	-	0.259
	IMI + GLI	20	0.103	0.340
14	IMI	13	0.249	-
	GLI	14	-	0.664
	IMI + GLI	12	0.228	0.570

Table S2. Structure of generalised linear models used for each response variable. † indicates the factor is random, as opposed to fixed. For mortality and food uptake assays, a separate analysis was performed for each rearing period. *In the accumulated mortality analysis, Cage was included in the saturated model to correct for overdispersion.

Assay	Response variable (distribution)	Factors in saturated model
Mortality	Accumulated mortality (binomial)	IMI presence, GLY presence, Season, Cage†*
Food uptake	Total individual uptake (Gamma)	IMI presence, GLY presence, Season
Sucrose responsiveness	Score (binomial)	TIU, IMI presence, GLY presence, Age, Cage†
Simple olfactory conditioning	CR during training (binomial)	TIU, IMI presence, GLY presence, Age, Trial, Bee†, Cage†
	CR in test (binomial)	TIU, IMI presence, GLY presence, Age, Cage†
Differential olfactory conditioning	DI during training(binomial)	TIU, IMI presence, GLY presence, Age, Trial pair, Bee†, Cage†
	DI in test (binomial)	TIU, IMI presence, GLY presence, Age, Cage†